caused an upregulation of SOX9 and enhanced extracellular matrix protein production.
Conclusions: Depletion of PHD2 resulted in greater HIF-2α levels, and therefore enhanced SOX9-induced cartilage matrix production compared to the levels normally found in hypoxia (1% oxygen) implying that PHD2 inhibition offers a novel means to promote the chondrocyte phenotype and enhance cartilage repair in vivo.

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CARTILAGE-SPECIFIC KNOCKOUT OF SITE-1 PROTEASE IN POSTNATAL MICE RESULTS IN AN ABNORMAL GROWTH PLATE WITH DISRUPTION OF HYPERTROPHIC CHONDROCYTE DIFFERENTIATION AND SUBSEQUENT CHONDRODYSPLASIA

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Purpose: Site-1 protease (S1P) is a proprotein convertase that plays an essential role in the processing of latent, membrane-bound transcription factors such as SREBPs and ATF6, to their active form. Cartilage-specific knockout of S1P in mice ($S1P^{cko}$) results in chondrodysplasia with complete lack of endochondral bone formation. These mice exhibited poor cartilage development with a drastic decrease of collagen type IIB (Col IIB) in the matrix. Most of the Col IIB appears trapped inside the cell. Ultrastructural analysis of the cartilage showed engorged and fragmented endoplasmic reticulum. $S1P^{cko}$ mice die during or very soon after birth and therefore the study was restricted to embryonic time-points. The goal of this study is to investigate the role of S1P in cartilage/endochondral bone development at various developmental stages in postnatal mice with the intent to identify and dissect S1P-regulated pathways fundamental to skeletal development.
Methods: $S1P^{f/f}$ mice (homozygous for the $S1P^{flox}$ allele with floxed S1P exon 2) were crossed with Col2-CreER(T) mice (positive for Col2-CreER(T) transgene where Cre recombinase-mutant estrogen receptor [ER(T)] fusion protein is active only in presence of tamoxifen) and the progeny back-crossed with $S1P^{f/f}$ to create $S1P^{f/f}$;Col2-CreER(T) mice. Cartilage-specific deletion of S1P in postnatal mice ($S1P^{cko\text{-}ER(T)}$) was achieved by injecting $S1P^{f/f}$;Col2-CreER(T) mice with tamoxifen at P1 (1 day old) or P7 (7 day old) timepoints and harvested a week or more later. Tamoxifen injections were done for some pregnant mice at E18.5. The cartilage/bone from $S1P^{cko\text{-}ER(T)}$ mice and its wild type (WT) littermates were studied by X-ray microtomograph (Micro-CT), histology, immunohistochemistry (IHC), immunofluorescence (IF), and in situ hybridization.
Results: $S1P^{cko\text{-}ER(T)}$ mice exhibit chondrodysplasia with severity depending on the timing of tamoxifen injection after birth. The younger the injected animals, the more pronounced the difference from its WT littermates, with tamoxifen injection of the mother at E18.5 being the most drastic. Micro-CT analysis of femurs and humeri showed that the bones in $S1P^{cko\text{-}ER(T)}$ mice were smaller with abnormal trabecular development. $S1P^{cko\text{-}ER(T)}$ mice appeared osteoporotic with drastically reduced trabecular bone (Fig. 1). Trabecular bone developed in an arbitrary fashion with individual trabeculae being thicker, with higher bone mineral density and with more spacing between them, than in WT. Histological analysis showed that chondrocyte hypertrophic differentiation was disrupted in $S1P^{cko\text{-}ER(T)}$ mice and further confirmed by a loss of type X collagen expressing cells in the growth plate. $S1P^{cko\text{-}ER(T)}$ mice injected at P1 did not develop a secondary ossification center; IHC and IF analysis of these mice demonstrated that Col IIB was trapped primarily inside cells which would otherwise differentiate to be hypertrophic in the secondary ossification center in WT.

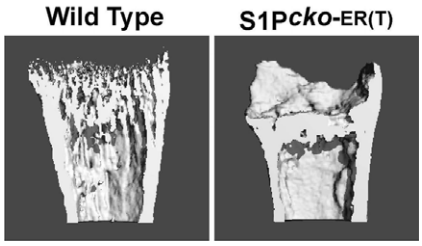

Figure 1. Micro-CT analysis of femurs from WT and $S1P^{cko\text{-}ER(T)}$ mice showing the osteoporotic nature of the femur in the mutant mice.

Conclusions: S1P is essential to endochondral bone development in postnatal mice. Bone lengthening is drastically reduced in $S1P^{cko\text{-}ER(T)}$ mice. The absence of type X collagen expressing cells in $S1P^{cko\text{-}ER(T)}$ mice suggests a defective growth plate diminishing bone elongation and implicates S1P in regulating hypertrophic differentiation in postnatal mice. As this is not seen in the $S1P^{cko}$ mice, S1P may have additional roles in postnatal mice toward skeletal development. The entrapment of Col IIB in the hypertrophic differentiation-arrested cells underscores a role for S1P in cartilage matrix homeostasis. The osteoporotic nature of the bone alludes to the importance of S1P in maintaining endochondral bone homeostasis. The phenotype in $S1P^{cko\text{-}ER(T)}$ mice is similar to that seen in humans with congenital achondroplasia and this attests to the fundamental requirement of S1P in mammalian skeletal development. Understanding this fundamental requirement should be useful in treating cartilage diseases and tissue engineering.

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POST-TRANSCRIPTIONAL REGULATION OF CYCLOOXYGENASE-2 EXPRESSION BY MICRORNA-199a* IN HUMAN OA CHONDROCYTES

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Purpose: MicroRNAs (miRNAs) are small non-coding RNAs that modify the properties of the cells by altering the translational profile of mRNAs to produce a proteome with unique functional properties. Interleukin-1β (IL-1β) mediated over expression of cyclooxygenase-2 (COX-2) contributes to the inflammation and joint degeneration in osteoarthritis (OA) patients. In this study we determined the posttranscriptional regulation of COX-2 by miRNAs in human OA and normal chondrocytes stimulated with IL-1β.
Methods: Chondrocytes were derived by enzymatic digestion following our standard protocol using cartilage from normal (trauma cases with no history of OA) and OA patients undergoing total joint arthroplasty. Isolated chondrocytes were stimulated with IL-1β (5 ng/ml) *in vitro*. Total RNA was prepared using the TRIZOL reagent and miRNAs were purified using the mirVANA system. Single stranded cDNA was synthesized using stem loop-specific primers and the expression of miRNAs of interest was quantified using TaqMan miRNA expression assay and their target mRNA was identified using bioinformatics. Transfection of chondrocytes with a 3'UTR reporter construct and pre-miRNAs was employed to verify the miRNA:mRNA interaction. Expression of COX-2 mRNA was determined by qRT-PCR using validated primers. Expression of COX-2 protein in OA chondrocytes transfected with pre-miRNAs and anti-miRNAs was determined by Western immunoblotting. Role of p38-MAPKs and NF-κB in the regulation of miRNAs and COX-2 expression in chondrocytes was evaluated using specific inhibitors. Data was analyzed using Origin 6.1 software package and $p<0.05$ was considered significant.
Results: We recently performed the miRNAs expression profile in human OA chondrocytes stimulated with IL-1β and discovered that the expression of several miRNAs, including miR-101_3, and miR-199a*, was suppressed significantly (>2 fold, $p<0.05$). Kinetic analysis showed a differential pattern of expression of miR-101_3, and miR-199a* miRNAs at 6 and 24 h post-stimulation with IL-1β. In human OA chondrocytes, expression of miR-101_3 was down regulated at 6 h (2.1-fold ± 1.0; n=7) but no significant change was observed at 24 h (n=11). In contrast, normal human chondrocytes showed down regulation of miR-101_3 (1.7-fold ± 0.53; n=3) at 24 h with no change at 6 h post-stimulation with IL-1β. Expression of miR-199a* was significantly down regulated in OA chondrocytes stimulated with IL-1β at 24 h (3.3-fold ± 1.5; n=11, $p<0.05$) but not at 6 h (0.94-fold ± 0.86; n=7, $p>0.05$). Importantly, in human OA chondrocytes stimulated with IL-1β expression of COX-2 protein was also found to be high at 24 h, compared to 6 h post stimulation and this inversely correlated with the expression of miR-199a*. Normal human chondrocytes also showed similar inverse correlation of COX-2 protein and miR-199a* expression at 6 h (1.1-fold ±0.023; n=3, $p>0.05$) and 24 h (2.2-fold ± 0.65; n=3, $p<0.05$) after stimulation with IL-1β. *In silico* analysis identified the sequences conserved in the 3'UTR of human COX-2 mRNA (NM_000963) complimentary to the seed sequence of miR-101_3, and miR-199a*. Over expression of miR-199a* in human OA chondrocytes inhibited the IL-1β-induced expression of COX-2 protein. On the other hand transfection with an inhibitor of miR-199a* enhanced the IL-1β-induced expression of COX-2 protein in human OA chondrocytes. Co-transfection of human OA chondrocytes with a luciferase reporter construct containing the 3'-UTR of human COX-2 mRNA and the miR-199a* mimic suppressed the luciferase enzyme activity significantly ($p<0.001$). No inhibition of luciferase enzyme activity was observed in OA chondrocytes transfected with either miR-199a* inhibitor or scrambled